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INTERNATIONAL/U.S. PATENT APPLICATION

A SYSTEMATIC APPROACH

TO MECHANISM-OF-RESPONSE ANALYSES

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A SYSTEMATIC APPROACH

TO MECHANISM-OF-RESPONSE ANALYSES

5 **CROSS-REFERENCES TO RELATED APPLICATIONS**
This application is related to U.S. provisional patent application

60/220,080, filed July 21, 2000 and claims priority to, and benefit of this application,

pursuant to 35 U. S. C. §119(e) and any other applicable statute or rule.

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15 **BACKGROUND OF THE INVENTION**
Functional genomics is a rapidly growing area of investigation, which

includes research into genetic regulation and expression, analysis of mutations that cause

changes in gene function, and development of experimental and computational methods

for nucleic acid and protein analyses. Proteomics has also emerged as a valuable tool for

20 determining the physiological basis for disease, and for examining the mechanisms of

drug action and toxicity. However, with the large numbers of nucleic acid and protein

sequences available for examination, selection of biological targets for the development

of potential new drug compositions must shift towards technology platforms that can add

additional value to the gene selection process, for example, by correlating a particular

25 molecular target with the underlying pathophysiology of a disease. There continues to be

a need to identify novel targets and drug compositions that are relevant to disease. The

present invention meets these and other needs by providing new methods for identifying

compositions having a desired activity, as well as methods for identifying organisms that

are sensitive or resistant to drug compositions.

30 **SUMMARY OF THE INVENTION**
The present invention provides methods for identifying new compositions

having a desired activity. The methods are based upon genetic response profiles

1

5 generated for an initial set of compositions, wherein at least one member of the set of
   compositions has been shown to have at least a first demonstrated activity and a second
   desired activity. The methods include the steps of providing the first set of compositions,
   determining a genetic response profile for each member composition, comparing the one
   or more component responses from the genetic response profile to the first demonstrated
10 activity and second desired activity of each member composition, thereby identifying a
   pattern of responses correlating to a decrease in the first demonstrated activity and an
   increase in the second desired activity; and screening a library of test compositions for the
   pattern of responses.

        In these methods, determining the genetic response profiles involves a)
15 providing a plurality of cell lines, b) treating each member of the plurality of cell lines
   with each member composition of the set of compositions; and c) detecting one or more
   responses to the member composition. The plurality of cell lines comprises at least one
   modified cell line which differs from a corresponding parent cell line in either the first
   demonstrated activity or the second desired activity. Optionally, the plurality of cell lines
20 includes both modified cell lines and parental cell lines. In one embodiment of the
   present invention, one or more of the cell lines are optimized for the analysis of a
   particular disease area of interest, such as cancer, inflammation, cardiovascular disease,
   diabetes, various infectious diseases, proliferative diseases, immune system disorders, or
   central nervous system disorders.

25   Optionally, the modified cell line differs from the corresponding parent
   cell line in the activity or concentration of a selected protein or nucleic acid, for example,
   in response to the addition of one or more agents or compositions. The plurality of cell
   lines can also be generated via a genetic selection process, giving rise to one or more cell
   lines which are, for example, drug resistant.

30   In a preferred embodiment of the present invention, the set of compounds
   used to generate the initial genetic response profile includes one or more drug
   compositions identified for treating the first demonstrated activity. The set of
   compositions can range, for example, from about 5 to about 50 compositions, or
   optionally, from about 10 to about 20 compositions. Optionally, the set of compositions
35 includes two or more analogous compounds.

        During the generation of the genetic response profile, the cell lines are
   treated with the member compounds. In one embodiment, treating each member of the

                                          2

5   plurality of cell lines involves administering varying concentrations of the plurality of
     compounds, thereby generating a dose-response. The cells are then examined using any
     of a number of broad scanning techniques, to measure the concentration or activity of at
     least one gene or gene product, in addition to the desired second activity (and optionally,
     the demonstrated first activity). For example, for measurement of RNA-type gene

10   products, the broad scanning technique(s) employed can include microarray analysis,
     differential display, EST screening, or combinations of these techniques. Alternatively,
     for the measurement of various proteins, the scanning techniques can include 2D-gel
     electrophoresis, LC mass spectrometry, and various immunoscreening techniques.
     Proteins of interest include, but are not limited to, signaling proteins, regulatory proteins,

15   pathway specific proteins, and receptor proteins. Optionally, flow cytometry and/or mass
     spectrometry can be employed, for example, in the detection of various responses.

          Detection of responses can also include detecting a change in any number
     of cellular or physical processes, including, but not limited to, cellular transcriptional
     activity, cellular translational activity, gene product activity, stability, abundance,

20   compartmentalization, or phenotypic endpoint. For example, assays including, but not
     limited to, one or more of an RNA transcription assay, a protein expression assay, a
     binding assay, a protein function assay, a phenotype-based cellular assay, a metabolic
     assay, a small molecule assay, an ionic flux assay, a reporter gene assay, a cell
     proliferation assay, an apoptosis assay, a cell adhesion assay, a cell invasion assay, a

25   calcium signaling assay, a cell cycling assay, a nitric oxide signaling assay, a receptor
     expression assay, or a gene promoter reporter assay, can be employed in the methods of
     the present invention.

          Comparative analysis are performed on the one or more responses, the first
     demonstrated activity and the second desired activity, to generate a pattern of responses

30   correlating to the first demonstrated activity and the second desired activity. The desired
     pattern is preferably a decrease in the first demonstrated activity, concomitant with an
     increase in the desired activity. Alternatively, the first demonstrated activity may stay at
     the same or similar level, while the desired activity is increased or amplified.
     Comparative analyses can be approached in any of a number of ways, including, but not

35   limited to, generating a graphical representation of the one or more responses over a
     plurality of time points, or performing mathematical calculations such as clustering

                                          3

5   analysis, multivariate analysis, analysis in n-dimensional space, principle component analysis, or difference analysis.

As a further step in the methods of identifying a new composition with a desired activity, a second set of compositions, or library of compositions, is screened by determining the genetic response profiles for member components. Optionally, the
10   genetic profile is determined in a manner similar to that used for the first set of compositions. However, the set of genetic responses determined need not be the same as those determined for the first set of composition; a selected subset of responses can be monitored.

The present invention also provides methods of identifying organisms that
15   are sensitive to treatment with a drug composition. The methods include the steps of: identifying a set of genetic response markers (e.g., a set of genes which correlate to expression response markers) of a biochemical process or disease state for which the drug composition is used as treatment; providing a plurality of cell lines, wherein the plurality of cell lines comprises at least one modified cell line that differs from a corresponding
20   parent cell line in at least one expression marker, or in its sensitivity to the drug composition; determining one or more genetic response profiles by a) treating each member of the plurality of cell lines with the drug composition; and b) monitoring the set of genetic response markers; comparing the one or more genetic response profiles to clinical data for a first population of organisms, thereby identifying a pattern of responses
25   correlating to sensitivity to treatment with the drug composition; and generating additional genetic response profiles for members of a second population of organisms and screening the additional genetic response profiles for the pattern of responses correlating to sensitivity, thereby identifying organisms that are sensitive to treatment with the drug composition. Optionally, the genetic response marker comprises a marker which
30   correlates to drug sensitivity, and the plurality of cell lines includes cell lines which are resistant to the drug treatment. The cell lines can be generated from a subset of cell lines used to identify the set of genes which correlate to the biochemical process (for example, apoptosis) or disease state (e.g., cancer).

As described in greater detail below, the methods provided herein provide
35   mechanisms for the a) determination of the most probable mechanism or mechanisms of action for a drug composition, b) identification of new compositions having a desired

4

5   activity, and c) identification of organisms that are sensitive (or resistant) to treatment
with a drug composition

DETAILED DISCUSSION

Before describing the present invention in detail, it is to be understood that
this invention is not limited to particular compositions or biological systems, which can,
10   of course, vary. It is also to be understood that the terminology used herein is for the
purpose of describing particular embodiments only, and is not intended to be limiting. As
used in this specification and the appended claims, the singular forms "a", "an" and "the"
include plural referents unless the content clearly dictates otherwise. Thus, for example,
reference to "a device" includes a combination of two or more such devices, reference to
15   "an analyte" includes mixtures of analytes, and the like.

Unless defined otherwise, all technical and scientific terms used herein
have the same meaning as commonly understood by one of ordinary skill in the art to
which the invention pertains. Although any methods and materials similar or equivalent
to those described herein can be used in the practice for testing of the present invention,
20   the preferred materials and methods are described herein.

DEFINITIONS

In describing and claiming the present invention, the following
terminology will be used in accordance with the definitions set out below.

A "genetic response profile" as used herein refers to a set of responses to a
25   stimuli, reflecting the biochemical events and changes occurring in a cell at a given point
in time (i.e. pre- or post- stimulation with, for example, a test composition).

The terms "plurality of cell lines" or "matrix of cell lines" refer to one or
more sets of cell lines used, for example, in the preparation of a set of genetic response
profiles. Exemplary pluralities of cell lines are described in, for example, PCT
30   application PCT/US01/08670, filed March 16, 2001, which is hereby incorporated by
reference in its entirety.

The term "biochemical pathway" is used herein to describe any
interrelated series of events or reactions; as such, this term is meant to encompass genetic
pathways (series of reactions leading to induction or reduction in gene expression) as well
35   as synthetic or catabolic pathways, metabolic pathways, catalytic pathways and the like.

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5 METHODS OF IDENTIFYING NEW COMPOSITIONS WITH DESIRED
ACTIVITIES

For many existing and novel therapeutics, the mechanism of cellular response is poorly understood. Even in cases where compounds are known to bind to a specific target, there may be secondary or tertiary binding events that are responsible for

10 the principal *in vivo* therapeutic mechanism. In addition, one or more secondary effects (e.g. "side effects") of some therapeutic compounds may constitute an additional desired activity, independent of the demonstrated activity for which the therapeutic compound was initially developed. By understanding how a set of compounds and/or compound analogues effect various genetic and cellular responses in a selected series of cell lines, it

15 is possible to correlate a set of responses with the desired activity (and optionally, without the demonstrated activity), thereby providing a screening mechanism for identifying, selecting, and/or optimizing compositions that produce the desired response profile or target a specific disease area of interest. Furthermore, this approach can be used to evaluate and anticipate the consequences of clinical use of the selected compound(s),

20 information that is potentially valuable for deciding whether or not to carry a compound into the clinic, or in aiding the FDA review process.

The present invention provides methods for identifying new compositions having one or more desired activities. The methods are based upon genetic response profiles generated for an initial set of compositions, where at least one member of the set

25 of compositions has been shown to have at least a first demonstrated activity and a second desired activity. By examining the patterns of genetic and cellular responses (i.e., the genetic response profiles) evoked by a first set of compositions having varying degrees of one or both activities, a preferred pattern of genetic responses can be formulated which corresponds to the desired activity, but not to the demonstrated activity. Additional sets

30 of compounds or compositions can then be screened for the desired genetic response profile. Further aspects of the methods of the present invention are described in greater detail in the following sections.

CELL LINES

The methods of the present invention are based upon responses generated

35 in a plurality of cell lines. The plurality of cell lines includes at least one modified cell line which differs from another cell line, optionally the parent line, in either the first demonstrated activity or the second desired activity. The differences in the cell lines

6

5   provide the means to identify and dissect one or more responses associated with each activity.

In one embodiment, one or more of the cell lines included in the plurality of cell lines differ in the concentration or activity of only one or a few nucleic acids and/or proteins, optionally leading to an altered activity level for either the first

10   demonstrated activity or the second desired activity. These pin-point differences simplify the process of identifying responses that correlate specifically to one or both activities. In another embodiment, the cell lines differ in the activity of multiple nucleic acids and/or proteins, some of which are associated with the first demonstrated activity and/or the second desired activity, while others are not. The responses generated by these lines can

15   also be used to identify and analyze the specific responses associated with each activity. Additional information can be obtained, for example, from the use of a larger set cell lines, and/or using scientific knowledge available from a number of sources including research databases and publications.

Potential member cell lines includes cell lines derived from, for example,

20   one or more different types of tissues or tumors, primary cell lines, cells which have been subjected to transient and/or stable genetic modification, and the like. Optionally, the cells are mammalian cells, for example murine, rodent, guinea pig, rabbit, canine, feline, primate or human cells. Alternatively, the cells can be of non-mammalian origin, derived, for example, from frogs, amphibians, or various fishes such as the zebra fish.

25   Cell lines which can be used in the methods of the present invention include, but are not limited to, those available from cell repositories such as the American Type Culture Collection (www.atcc.org), the World Data Center on Microorganisms (http://wdcm.nig.ac.jp), the European Collection of Animal Cell Culture (www.ecacc.org) and the Japanese Cancer Research Resources Bank (http://cellbank.nihs.go.jp). These

30   cell lines include, but are not limited to, HeLa cells, COS cells, lung carcinoma cell lines including squamous cell carcinoma cell lines (such as LK-2, LC-1, EBC-1, and NCI-H157), large cell carcinoma cell lines (such as H460 and H1299), small-cell carcinoma cell lines (such as H345, H82, H209, and N417); adenocarcinoma cell lines (such as A549, H322, H522, H358, H23 and RERF-LC-MS); fibrosarcoma cell lines (such as

35   HT1080); prostrate cancer cell lines (e.g., PC3, DU145, LNCaP, MDA-PCa 2a, MDA-PCa 2b, ARCaP) and other cell lines commonly used by one of skill in the art (for example: 293, 293Tet-Off, CHO-AA8 Tet-Off, MCF7, MCF7 Tet-Off, LNCap, T-5,

7

5   BSC-1, BHK-21, Phinx-A, 3T3, ZR 75-1, HS 578-T, DBT, Bos, CV1, L-2, RK13,
HTTA, HepG2, BHK-Jurkat, Daudi, RAMOS, KG-1, K562, U937, HSB-2, HL-60,
MDAHB231, C2C12, HTB-26, HTB-129, HPIC5, A-431, CRL-1573, 3T3L1, Cama-1,
J774A.1, HeLa 229, PT-67, Cos7, OST7, HeLa-S, THP-1, and NXA.) Additional cell
lines for use in the methods and kits of the present invention can be obtained, for
10   example, from cell line providers such as Clonetics Corporation (Walkersville, MD;
www.clonetics.com).

        The number of cell lines employed in the methods of the present invention
will vary based upon a number of factors, such as the desired activity, the disease area of
interest, and the number of relevant cell lines available. Additional considerations
15   include, but are not limited to, the representation of diverse cell types (for example, the
use of diverse cancer cell types for screening of cancer inhibitory compounds), previous
usage in the study of similar compounds, and sensitivity or resistance to drug treatment.
The plurality of cell lines can range in number from, for example, about two cell lines to
about 5, about 10, about 15, about 20, or more cell lines (to as many as about 10^3 or about
20   10^4 cell lines). Optionally, the methods are performed in a high throughput, multiwell
format.

                Modified Cell Lines
        The plurality of cell lines employed in the methods of the present
invention optionally includes both modified cell lines and parental cell lines. The
25   modified cells and optional parental cells typically differ by one or more modifications
that have been made to at least one biochemical or genetic pathway. Thus, in some
embodiments of the methods of the present invention, the modified cell line differs from
the corresponding parent cell line in the activity or concentration of a selected protein or
nucleic acid. Alternatively, the differences between parental cell and modified daughter
30   cell may arise from multiple sites or sources of dissimilarity. Any combination of
singular-modified cell, multiply modified cell and parental cell can be included in the
plurality of cell lines of the present invention.

        The difference between modified (daughter) cell line and parental (e.g.
wild type) cell line can arise, for example, from changes in the "functional activity" of at
35   least one biological molecule, for example, a protein or a nucleic acid. A difference in
the functional activity of a biological molecule refers to an alteration in an activity and/or
a concentration of that molecule, and can include, but is not limited to, changes in

                                        8

5   transcriptional activity, translational activity, catalytic activity, binding or hybridization activity, stability, abundance, transportation, compartmentalization, secretion, or a combination thereof. The functional activity of a biological molecule can also be affected by changes in one or more chemical modifications of that molecule, including but not limited to adenylation, glycosylation, phosphorylation, acetylation, methylation,

10   ubiquitination, and the like.

         The alteration in activity or concentration of the at least one biological molecular can arise from a number of treatments of the parental cell line. Furthermore, the alteration can be a permanent change (e.g., a mutation or an irreversible structural modification) or it can be a temporary response to a stimulation. Examples of stimulatory

15   agents, chemicals and treatments which can be used to generate the modified cell lines of the present invention include, but are not limited to, oxidative stress, pH stress, pH altering agents, DNA damaging agents, membrane disrupters, metabolic blocking agents, and energy blockers. Additionally, cellular perturbation may be achieved by treatment with chemical inhibitors, cell surface receptor ligands, antibodies, oligonucleotides,

20   ribozymes and/or vectors employing inducible, gene-specific knock in and knock down technologies.

         The identity and use of stimulatory agents, chemicals and treatments are known to one of skill in the art. Examples of DNA damaging agents include, but are not limited to, intercalation agents such as ethidium bromide; alkylating agents such as

25   ethylnitrosourea and methyl methanesulfonate; hydrogen peroxide; UV irradiation, and gamma irradiation. Examples of oxidative stress agents include, but are not limited to, hydrogen peroxide, superoxide radicals, hydroxyl free radicals, perhydroxyl radicals, peroxyl radicals, alkoxyl radicals, and the like. Examples of metabolic blocking and/or energy blocking agents include, but are not limited to, azidothymidine (AZT), ion (e.g.

30   Ca^{++}, K^+, Na^+) channel blockers, α and β adrenoreceptor blockers, histamine blockers, and the like. Examples of chemical inhibitors include, but are not limited to, receptor antagonists and inhibitory metabolites/catabolites (for example, mavelonate, which is a product of and in turn inhibits HMG-CoA reductase activity).

         In some embodiments of the present invention, the alteration in activity or

35   concentration of a biomolecule is evoked in the modified cell in response to the presence of one or more modification agents. Exemplary agents include, but are not limited to, compositions that modify DNA structure (e.g., ethylnitrosourea, quinoxaline antibiotics),

9

5   compositions that affect DNA activity, compositions that alter protein expression and/or affect protein functional activity (e.g. by inducing or inhibiting the activity), or compositions that induce a combination of these effects. For example, a number of compounds that alter DNA activity do so by inducing or inhibiting transcription or translation of the nucleic acid sequence, or by affecting splicing processes or

10   transcriptional modifications. Alternatively, certain compounds alter protein expression by modifying or interfering with translation, transportation or post-translational modification processes.

Additional agents which can be used to generate modified cell lines include, but are not limited to, antisense agents, ribozymes, receptor ligands (which can

15   either induce or inhibit a range of cellular events), antigens, antibodies, and the like. For example, antisense oligonucleotides can be used to alter gene function, validate gene targets, and even as therapeutic treatments (Baker et al. "Discovery and analysis of antisense oligonucleotide activity in cell culture" Methods 2001 Feb 23:191-8; Koller et al. "Elucidating cell signaling mechanisms using antisense technology" Trends Pharmacol

20   Sci 2000 Apr 21:142-8). Alternatively, ribozymes can be used to down-regulate (by RNA cleavage) or repair (by RNA trans-splicing)gene expression and elicit specific changes in gene/protein expression (see for example, Rossi "Ribozyme therapy for HIV infection" Adv Drug Deliv Rev 2000 Oct 44:71-8; Phylactou "Ribozyme and peptide-nucleic acid-based gene therapy" Adv Drug Deliv Rev 2000 Nov 44:97-108). Peptide

25   nucleic acid (PNA) technology can also be used to alter genetic function and produce modified cells for use in the present invention (Nielsen "Peptide nucleic acid: a versatile tool in genetic diagnostics and molecular biology" Curr Opin Biotechnol 2001 Feb;12(1):16-20; Nielsen "Antisense peptide nucleic acids" Curr Opin Mol Ther 2000 Jun;2(3):282-7). Various antibiotics (lexistropsin, luzopeptin , triostin A, distamycin,

30   echinomycin, mitomycin, bleomycin, and other quinoxaline antibiotics), antigens (endotoxins, lectins) and receptor ligands (retinol, estradiol, various growth factors) can also initiate cellular or metabolic changes leading to modified cell lines for use in the present invention.

In one embodiment of the present invention, one or more of the cell lines

35   are optimized for the analysis of a particular disease area of interest prior to use in the plurality of cell lines. Utilization of one or more optimized cell lines or sets of cell lines potentially enhances the screening of compounds for a related treatment. Optionally, the

5   collection of cells can be selected and/or optimized for the analysis of a particular
     biological or genetic pathway, or for cells that exhibit traits relevant to specific disease
     phenotypes or areas of interest. Disease areas of interest of the present invention include,
     but are not limited to, cancer, inflammation, cardiovascular disease, diabetes, infectious
     disease, proliferative diseases, immune system disorders (such as AIDS), and central
10   nervous system disorders (for example, Alzheimer's disease and Parkinson's disease).
     However, additional areas of clinical interest could easily be determined by one of skill in
     the art. If a target molecule for a specific disease is known, the component cell lines in
     the plurality can be selected for modifications that focus on this particular molecule and
     the pathways in which it participates. Alternatively, the cell lines can be selected for
15   modifications made in one or more "marker" molecules that correlate to a disease-related
     pathway of interest.

          In some embodiments of the present invention, the plurality of cell lines
     includes member cell lines which have been generated via a process of genetic selection.
     Genetic selection, as it is being considered here, is the process of altering the genetic
20   profile, optionally in a directed way, for a cell or whole organism. In one approach, the
     process typically involves taking the cell through a number of generations of cell cycle.
     During the replication process genetic mutations occur, either naturally or induced by one
     or more mutagenic agents (e.g. UV light or a DNA damaging compound, for example,
     ethyl-nitroso-urea (ENU)). Some of these mutations lead to alteration in the activity or
25   concentration of different RNAs and proteins as monitored in the genetic response
     profile. Alternatively, mutagenesis can be induced in a more controlled manner (i.e.,
     single nucleotide substitutions, multiple nucleotide substitutions, and insertion or deletion
     of regions of the nucleic acid sequence), such as by site directed mutagenesis, shuffling,
     or recursive recombination.

30   A variety of mutagenesis protocols, such as viral-based mutational
     techniques, homologous recombination techniques, gene trap strategies, inaccurate
     replication strategies, and chemical mutagenesis, are available and described in the art.
     These procedures can be used separately and/or in combination to produce modified cell
     lines for use in the methods of the present invention. See, for example, Amsterdam et al.
35   "A large-scale insertional mutagenesis screen in zebrafish" Genes Dev 1999 Oct 13:2713-
     2724; Carter (1986) "Site-directed mutagenesis" Biochem. J. 237:1-7; Crameri and
     Stemmer (1995) "Combinatorial multiple cassette mutagenesis creates all the

5   permutations of mutant and wildtype cassettes" BioTechniques 18:194-195; Inamdar
     "Functional genomics the old-fashioned way: chemical mutagenesis in mice" Bioessays
     2001 Feb 23:116-120; Ling et al. (1997) "Approaches to DNA mutagenesis: an
     overview" Anal Biochem. 254(2): 157-178; Napolitano et al. "All three SOS-inducible
     DNA polymerases (Pol II, Pol IV and Pol V) are involved in induced mutagenesis"
10   EMBO J 2000 Nov 19:6259-6265; and Rathkolb et al. "Large-scale N-ethyl-N-
     nitrosourea mutagenesis of mice--from phenotypes to genes" Exp Physiol 2000 Nov
     85:635-44.   Furthermore, kits for mutagenesis and related techniques are also available
     from a number of commercial sources (see, for example, Stratagene
     (http://www.stratagene.com/vectors/index2.htm), Clontech
15   (http://www.clontech.com/retroviral/index.shtml), and the Gateway cloning system from
     Invitrogen (http://www.invitrogen.com). General texts which describe molecular
     biological techniques useful in the generation of modified cell lines, including
     mutagenesis, include Berger and Kimmel, Guide to Molecular Cloning Techniques,
     Methods in Enzymology, volume 152 Academic Press, Inc., San Diego, CA; Sambrook et
20   al., Molecular Cloning - A Laboratory Manual (2nd Ed.), volumes 1-3, Cold Spring
     Harbor Laboratory, Cold Spring Harbor, New York, 1989; and Current Protocols in
     Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between
     Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through
     2000)).

25   Selection of Modified Cell Lines
                 The selection process involves the use of different experimental techniques
     to select those cells which have mutated in the desired manner. For example, the
     selection process can include, but is not limited to: identifying cells that survive and/or
     continue to grow under different environments, stresses and/or stimulation; cells that have
30   increased or decreased expression of a particular protein that can be used to sort or
     separate cells with the altered protein levels, (e.g. using flow cytometry to sort cells that
     are over expressing a particular cell surface receptor); and cells that have an altered
     physical phenotype that can be identified and selected, e.g. cells arrested in a particular
     cycle phase, cells that have altered ability to invade a barrier or translocate, cells that have
35   a different shape, or have or have not differentiated into a different cell type). Numerous
     additional selection methods are known to one of skill in the art and can be employed to
     provide cell lines for use in the methods of the present invention.

5   The plurality of cell lines employed in the methods of the present invention optionally include resistant cell lines. In certain diseases, e.g. cancer, it is as important to understand mechanisms of resistance as well as mechanisms of action of a therapeutic composition. Selection of appropriate cell lines for use in the methods of the present invention will influence both the identification of novel compositions for the

10 treatment (or prevention) of the disease state, as well as any analysis of cellular mechanisms that potentially confer drug resistance. Optionally, one or more existing disease model cell lines (e.g., modified cell lines or parental cell lines) undergo a selection process to create one or more drug resistant cell lines. The resistant cell lines can be analyzed and/or isolated using various techniques known to one of skill in the art;

15 for example, flow cytometry can be used to sort through and collect cells that carry traits of drug resistance. A comparative analysis between non-resistant and resistant cell lines is optionally performed to identify differences in genetic and cellular responses, thereby identifying the cellular elements responsible for resistance. This information can be used, for example, to anticipate potential problems in the clinic, or to design or identify new

20 compounds that bypass these mechanisms of resistance.

    As another example, a cell survival selection process can be used to screen for modified cells that have been genetically altered to resist compositions that induce apoptosis. In one approach to generation of apoptosis-resistant cells, a dose response analysis is performed for every member cell line and composition. Concentrations of

25 drugs are tested to identify the optimum dose(s) to maximize killing in a specified length of time, for example, two weeks. Using the optimum dose, cell colonies are treated and selected over a second period of time (e.g., 3 to 4 weeks). Alternatively, modified cell lines can also be generated with varying doses of chemicals. The end product is a series of cell lines with various levels of drug resistance that can be directly compared with their

30 drug sensitive parents.

<div align="center">Knockin, Knockout, and Knockdown Cell Lines</div>

    Cell lines carrying specific gene knockdowns or knockins provide excellent model systems for analyzing biochemical and genetic mechanisms, particularly when the only difference among the cell lines is the alteration in the level and/or activity

35 of a single protein or nucleic acid. These pinpoint genetic alterations provide an efficient means to decipher the roles played by various nucleic acids and/or proteins within the biochemical pathways in which they participate.

<div align="center">13</div>

5   For example, HeLa cell lines can be finely altered to, in one circumstance,
over express the p53 protein, and in another circumstance to under express c-myc. These
alterations involve the insertion of exogenous elements that enable the overproduction of
a protein (knockin) or reduction in the production of a constitutive protein (knockdown)
within the cell. Alternatively, the targeted gene can be prevented from expressing any

10   protein (knockout) via a number of processes, including deletion of the gene or
transcription promoting elements for the gene at the DNA level within the cell. Knockout
modifications generally involve modification of the gene or genes within the genome
(see, for example, Gonzalez (2001) "The use of gene knockout mice to unravel the
mechanisms of toxicity and chemical carcinogenesis" Toxicol Lett 120:199-208).

15   Knockdown modifications are typically achieved by either treatment with an exogenous
agent (e.g. antisense or ribozyme) or by insertion into the genome of one or more vectors
expressing a product that hybridizes to nucleic acid. The target nucleic acid is commonly
RNA, although DNA molecules can also be targeted. Furthermore, knockouts can be
either heterozygous (e.g. inactivating only one copy of the gene) or homozygous

20   (inactivating both copies of the gene). One exemplary database of mouse knockouts can
be found at http://research.bmn.com (the BioMedNet mouse knockout and mutation
database).

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25   mice to unravel the mechanisms of toxicity and chemical carcinogenesis" Toxicol Lett
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knockouts can be found at http://research.bmn.com (the BioMedNet mouse knockout and
mutation database).

          Once a genetic response profile has been developed for a desired activity

35   or biological system, gene-specific knockdowns can be created to specifically perturb
principal target molecules within the system. Knockdowns are typically utilized in two
ways. The first use is to confirm that a targeted knockdown leads to the same genetic and

14

5   phenotypic response as is caused by a model or principal compound (e.g., the
       composition that evokes the first demonstrated activity and the second desired activity).
       The second common application is the use of stable knockdowns to turn off principal
       pathways with the cells. These cell are then treated with the compositions and screened
       to determine which pathways are primary to the phenotypic response stimulated by the
10   compound. A knock down within the key pathway will block the mechanism of action
       and show an altered genetic response profile, thereby confirming the primary mechanism.

              Thus, the plurality of cell lines employed in the present invention can
       include a combination of parental or wildtype cells, singular-modification cells, multiply-
       modified cells, resistant cells, cells optimized for a particular disease state, and the like.
15   Further details regarding the generation and use of pluralities of cell lines can be found in
       PCT application PCT/US01/08670 (Monforte et al.), filed March 16, 2001.

       COMPOSITIONS AND ACTIVITIES
              The methods of the present invention include the step of providing a first
       set of compositions, wherein at least one member of the first set of compositions
20   comprises at least a first demonstrated activity and a second desired activity. In addition,
       the methods include the step of screening a second set of compositions for the pattern of
       responses, thereby identifying a new composition with the desired activity. The genetic
       response profiles generated upon treatment of the plurality of cell lines with the first set
       of compositions are compared to the first demonstrated activity and second desired
25   activity of each member composition, to identify a desired pattern of responses
       correlating to an increase in the second desired activity. Preferably, pattern of responses
       also correlates to a decrease (or at minimum, no change in) the first demonstrated activity.

              In a preferred embodiment of the present invention, the set of compounds
       used to generate the initial genetic response profile includes one or more drug
30   compositions identified for treating the first demonstrated activity. The set of
       compositions can range, for example, from about 5 to about 50 compositions, or
       optionally, from about 10 to about 20 compositions.

              Optionally, selection of the compounds that are used for generation of the
       initial genetic response profiles (or for screening of compositions for secondary desired
35   activities) is made based on literature and knowledge of experts in the field of interest. In
       order to take full advantage of the comparative analysis approach to discerning

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5   mechanism of response for a drug or composition and identifying new compositions, it is useful to analyze a selection of compositions including, but not limited to, a range of therapeutics (either approved or currently in clinical trials), therapeutic candidates, research chemicals, libraries of synthetic compositions, natural or biological compounds, herbal compositions, and other chemicals that potentially interact with one or more target

10   molecules or that appear to drive cells to a comparable phenotype(s).

        As is appreciated by one skilled in the art, the number of classes of compounds and/or compound analogues (optionally associated with a first demonstrated activity) that can be examined for secondary (desired) activities is extensive, and includes, but is not limited to, the following groups of compounds: ACE inhibitors; anti-

15   inflammatory agents; anti-asthmatic agents; antidiabetic agents; anti-infectives (including but not limited to antibacterials, antibiotics, antifungals, antihelminthics, antimalarials and antiviral agents); analgesics and analgesic combinations; apoptosis inducers or inhibitors; local and systemic anesthetics; cardiac and/or cardiovascular preparations (including angina and hypertension medications, anticoagulants, anti-arrhythmic agents,

20   cardiotonics, cardiac depressants, calcium channel blockers and beta blockers, vasodilators, and vasoconstrictors); chemotherapies, including various antineoplastics; immunoreactive compounds, such as immunizing agents, immunomodulators, immunosuppressives; appetite suppressants, allergy medications, arthritis medications, antioxidants, herbal preparations and active component isolates; neurologically-active

25   agents including Alzheimers and Parkinsons disease medications, migraine medications, adrenergic receptor agonists and antagonists, cholinergic receptor agonists and antagonists, anti-anxiety preparations, anxiolytics, anticonvulsants, antidepressants, anti-epileptics, antipsycotics, antispasmodics, psychostimulants, hypnotics, sedatives and tranquilizers, and the like. One advantage to generating genetic response profiles for a

30   defined class of compounds is that the compounds have already been through preclinical and/or clinical evaluation for the demonstrated activity, which provides support for and potentially speeds the process for approval for a second indication (the desired activity).

GENETIC RESPONSE PROFILES

        In the methods of the present invention, determining the genetic response

35   profiles involves a) providing a plurality of cell lines, b) treating each member of the plurality of cell lines with a composition; and c) detecting one or more responses to the

16

5   member composition. The compositions can be a member of the first set of compositions (i.e., during generation of the genetic response profile), or the composition can come from the second set of compositions being screened. Thus, a similar procedure can be employed in screening a library of compositions, although the screening step is not limited to repeating the same process as was previously used to generate the genetic
10   response profiles.

During the generation of the genetic response profile, the cell lines are treated with the member compounds and one or more genetic, biochemical or cellular responses are monitored. For example, changes in any number of cellular or physical processes, including, but not limited to, cellular transcriptional activity, cellular
15   translational activity, gene product activity, stability, abundance, compartmentalization, or phenotypic endpoint, can be included in the genetic response profile. For example, assays including, but not limited to, one or more of an RNA transcription assay, a protein expression assay, a binding assay, a protein function assay, a phenotype-based cellular assay, a metabolic assay, a small molecule assay, an ionic flux assay, a reporter gene
20   assay, a cell proliferation assay, a cell viability assay, an apoptosis assay, a cell adhesion assay, a cell invasion assay, a calcium signaling assay, a cell cycling assay, a nitric oxide signaling assay, a receptor expression assay, or a gene promoter reporter assay, can be employed in the generation of the genetic response profiles of the present invention. The responses can be measured at either a single timepoint or over a plurality of timepoints.
25   Optionally, at least one measurement is collected prior to treatment with the member composition.

The set of genes or gene products selected for inclusion in a given response profile can be selected, for example, by scanning the literature or by performing empirical studies. Preferably, the selected gene or gene products are a) expressed at
30   detectable levels within the plurality of cell lines, and b) are likely to change as a result of exposure to one or more member compositions. Two types of genes (or their respective gene products) are typically monitored during generation of the genetic response profile: genes that are empirical responders (i.e. marker genes) and genes that are known or suspected to be involved in the pathways or disease area of interest. Optionally, one or
35   more genes known to be affected by at least one composition in the set of compositions are monitored (e.g., a positive control). For the sake of experimental efficiency and to

17

5   optimize the gene set, an initial set of experiments can be performed on both the untreated
     cell lines and a set of treatments.

          RNA and proteins isolated from this small set of samples is analyzed using
     a number of broad scanning techniques as described below. From this analysis, as well as
     optional literature data, sets of genes/gene products (e.g. between about 10 and about 20,
10   about 50, about 100 or about 1000) are selected for response profiling. Protein and
     nucleic acid sequences that can be monitored in the methods of the present invention
     include, but are not limited to, those listed with the National Center for Biotechnology
     Information (www.ncbi.nlm.nih.gov) in the GenBank® databases, and sequences
     provided by other public or commercially-available databases (for example, the NCBI
15   EST sequence database, the EMBL Nucleotide Sequence Database; Incyte's (Palo Alto,
     CA) LifeSeq[TM] database, and Celera's (Rockville, MD) "Discovery System"[TM] database).
     For example, proteins that can be monitored (e.g., as part of the genetic response profile)
     in the plurality of cell lines used in the present invention include, but are not limited to,
     signaling proteins, regulatory proteins, pathway specific proteins, receptor proteins, and
20   other proteins involved in one or more biochemical pathways. Nucleic acids that can be
     monitored include, but are not limited to, DNA, genomic DNA, BAC or YAC constructs,
     viral DNA, plasmid DNA or other vectors, tRNA, rRNA, mRNA, guide RNA, snRNA
     molecules, snoRNA molecules, and hnRNA molecules.

          The genetic response profile will be compared to the first demonstrated
25   activity and second desired activity of the member compositions, to generate a desired
     profile best corresponding to the desired activity. The demonstrated first activity includes
     any of a number of activities, such as anti-inflammatory, anti-infective, analgesic, anti-
     hypertensive, antidepressant, immunoreactive, vaso-active and the like. Second desired
     activities of interest include, but are not limited to, antiproliferative, antineoplastic, or
30   anticancer activity.

          Detection Methods

          In one embodiment of the present invention, treating each member of the
     plurality of cell lines involves administering varying concentrations of the plurality of
     compounds, thereby generating a dose-response. The cells are then examined using any
35   of a number of broad scanning techniques, to measure the concentration or activity of at
     least one gene or gene product, in addition to the desired second activity (and optionally,
     the demonstrated first activity).

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5   A number of different detection methods can be used to visualize and
monitor the cellular responses as they occur following exposure of the plurality of cell
lines to the set of compositions. Such methods include, but are not limited to, RNA
transcription assays, protein expression assays, protein function assays, phenotype-based
cellular assays, metabolic assays, small molecule assays, ionic flux assays, reporter gene
10   assays, membrane alteration/disruption assays, intercellular signaling assays, selective
sensitivity-to-invasion assays, or a combination thereof. Many of these methodologies
and analytical techniques can be found in such references as Current Protocols in
Molecular Biology, F.M. Ausubel et al., eds., (a joint venture between Greene Publishing
Associates, Inc. and John Wiley & Sons, Inc., supplemented through 1999), Enzyme
15   Immunoassay, Maggio, ed. (CRC Press, Boca Raton, 1980); Laboratory Techniques in
Biochemistry and Molecular Biology, T.S. Work and E. Work, eds. (Elsevier Science
Publishers B.V., Amsterdam, 1985); Principles and Practice of Immunoassays, Price and
Newman, eds. (Stockton Press, NY, 1991); and the like.
          For example, changes in nucleic acid expression can be determined by
20   polymerase chain reaction (PCR), ligase chain reaction (LCR), Qβ-replicase
amplification, nucleic acid sequence based amplification (NASBA), and other
transcription-mediated amplification techniques; differential display protocols;
microarray analysis, EST screening, analysis of northern blots, enzyme linked assays,
and the like. Examples of these techniques can be found in, for example, PCR Protocols
25   A Guide to Methods and Applications (Innis et al. eds) Academic Press Inc. San Diego,
CA (1990).
          Alternatively, the expression pattern of genes can be rapidly analyzed as
described by Wang et al. (Nucleic Acids Research (1999) vol. 27, pages 4609-4618).
This technique employs PCR amplification of cDNAs which have been cleaved by
30   frequently-cutting endonucleases, such as *Dpn*II and *Nla*III, and primed with defined
sequences prior to amplification.
          Another method for detecting molecular events within the plurality of cell
lines utilizes real-time PCR for DNA and rtPCR for RNA, using, for example, FRET
(fluorescence resonance energy transfer) in TaqMan® (Applied Biosystems Inc.) or
35   molecular beacon assays. The FRET technique utilizes molecules having a combination
of fluorescent labels which, when in proximity to one another, allows for the transfer of

5   energy between labels (see, for example, X. Chen and P.-Y. Kwok, (1997) <u>Nucleic Acid</u> <u>Research</u> vol. 25, pp. 2347-2353).

For the measurement of various proteins, the scanning techniques can include 2D-gel electrophoresis, LC mass spectrometry, and various immunoscreening techniques. Optionally, the responses of the plurality of cell lines can be monitored by
10   fluorescence activated cell sorting, or FACS. A wide variety of flow-cytometry methods have been published. For a general overview of fluorescence activated flow cytometry see, for example, Abbas et al. (1991) <u>Cellular and Molecular Immunology</u>, W.B. Saunders Company; Coligan et al. (eds)(1991) <u>Current Protocols in Immunology, and</u> <u>Supplements</u>, John Wiley and Sons, Inc. (New York); and Kuby (1992) <u>Immunology,</u>
15   W.H. Freeman and Company,. Fluorescence activated cell scanning and sorting devices are available from several companies, including, e.g., Becton Dickinson and Coulter.

Alternatively, high throughput screening systems utilizing microfluidic technologies, available, for example, from Agilent/Hewlett Packard (Palo Alto, CA) and Caliper Technologies Corp. (Mountain View, CA) could be employed for detecting the
20   response(s) generated in the plurality of cell lines. The Caliper Lab Chip™ technology uses microscale microfluidic techniques for performing analytical operations such as the separation, sizing, quantification and identification of nucleic acids (for further information, see www.calipertech.com).

Generation of Profiles
25   For each cell line and each member composition, a series of experiments can optionally be performed to establish the optimal dosage and time point(s) for measuring response. A dose response study is performed with each compound using one or more of the genetic and/or phenotypic assays described above as the measurable endpoint. Time point(s) and dose level(s) are selected based on these studies.
30   Observation of cellular events as they occur over time and in response to one or more stimuli provides a dynamic view of the biomolecular activity of the cell. These cellular events, or responses, are evaluated and recorded for comparison. This is achieved by collecting the plurality of data points representing information related to the plurality of cell lines and the one or more responses of the cellular system to the at least
35   one stimulus.

For each experiment performed, the plurality of data points is gathered into a database and used to generate the genetic response profile for the corresponding cell

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5   line. The plurality of data points representing the cellular responses upon exposure to the composition being tested can be linear or nonlinear. In one embodiment of the present invention, determining a genetic response profile for each member composition consists of a) selecting a first cell line from the plurality of cell lines; b) evaluating at least one response, and optionally multiple responses; c) recording the evaluation of the at least one

10   response; and d) repeating these steps for additional cell lines in the plurality of cell lines. In another embodiment of the method of the present invention, the evaluating and recording of information is performed on the entire plurality of cell lines simultaneously. During the recording step, the response (or responses) generated for each cell line are entered into a profile database for further analysis. The entire set of cell lines can be

15   evaluated for response to a stimulus, or a subset of the set of cell lines can be examined.

Generation of genetic response profiles for each member composition versus the plurality of cell lines generally results in a large quantity of data reflecting information related to the cell types used and the responses measured for the plurality of cell lines. In one embodiment of the method of the present invention, the plurality of data

20   points is entered as character strings, or as descriptors, into a database. The character strings or descriptors can be used to encode include any relevant information derived from or detected within the plurality of cell lines, including any physical characteristics, activities, or other information related to the cell types used and the responses detected. In general, the database is embodied in a computer or computer readable medium and can

25   be accessed by a user and/or integrated system.

Genetic analysis is optionally complemented with phenotypic analysis of the cells, to build a model of how the cell systems respond to exposure to the set of compositions. A variety of phenotypic data can be acquired during the step of determining a genetic response profile for each member composition of the first set of

30   compositions, including, but not limited to, data related to proliferation, differentiation, apoptosis, cell adhesion, cell invasion, calcium signaling, cell cycling, nitric oxide signaling, receptor expression, gene promoter reporter, cell-cell interaction, cell matrix interaction, cell histology, pathology and other endpoints known to one with skill in the art. The employment of certain types of readout methodologies (e.g. microscopy, flow

35   cytometry, and bioselection) enables partition or selection of subpopulations of cells that can be further profiled for unique traits including altered drug resistance or sensitivity.

COMPARATIVE ANALYSES

       Comparative analysis are performed on the one or more responses, the first demonstrated activity and the second desired activity, to generate a pattern of responses correlating to the first demonstrated activity and the second desired activity. The desired pattern is preferably an increase in the desired activity, concomitant with a decrease in the

10 first demonstrated activity. Alternatively, the first demonstrated activity may stay at the same or similar level, while the desired activity is increased or amplified. Comparative analyses can be approached in any of a number of ways, including, but not limited to, generating a graphical representation of the one or more responses over a plurality of time points, or performing mathematical calculations such as clustering analysis, multivariate

15 analysis, analysis in n-dimensional space, principle component analysis, or difference analysis.

       Different experimental outcomes are compared by the similarity of the pattern of response profiles generated. This similarity is revealed using, for example, clustering analysis. A number of clustering algorithms are commonly used for this type

20 of study [see *Clustering Algorithms*, JA Hartigan, Wiley, NY 1975]. The comparisons between profiles can be performed at the level of individual genes, clusters of genes known to be involved in specific pathways or mechanisms, individual cell lines, or for the entire experimental data set. For example, for each experimental pair, e.g. two different composition treatment sets, a distance metric can be defined as $D = 1 - \rho$, where ρ is the

25 correlation coefficient between the expression profiles. The value of D indicates the level of similarity between two experimental pairs. In this manner, a matrix can be created wherein chemicals producing similar profiles closely cluster, i.e. D is small, and those with divergent profiles will have large D values. This type of analysis can reveal, for example, similarities in the mechanism of response of various chemicals. Furthermore,

30 analysis among similar cell types and between different cell types is used to determine what cell, tissue, organ or tumor types may be more or less vulnerable when exposed to a given chemical.

       In order to ascertain whether the observed changes in response profiles of the treated cell lines are significant, and not just a product of experimental noise or

35 population heterogeneity, an estimate of a probability distribution is optionally constructed for each genetic and phenotypic endpoint in each cell line. Construction of the estimated population distribution involves running multiple independent experiments

5   for each treatment, e.g. all experiments are run in duplicate, triplicate, quadruplicate or
       the like.

              The genetic response information is evaluated and the one or more
       responses from the genetic response profile are compared to the first demonstrated
       activity and second desired activity of each member composition. Analysis of the data
10   involves the use of a number of statistical tools to evaluate the measured responses and
       changes based on type of change, direction of change, shape of the curve in the change,
       timing of the change and amplitude of change. This information can be used to perceive
       and interpret the impact that alterations, ranging from a "minor" change in a single
       nucleotide to major permutations in one or more metabolic pathway, can have on the
15   biological systems network as a whole.

              Multivariate statistics, such as principal components analysis (PCA), factor
       analysis, cluster analysis, n-dimensional analysis, difference analysis, multidimensional
       scaling, discriminant analysis, and correspondence analysis, can be employed to
       simultaneously examine multiple variables for one or more patterns of relationships (for a
20   general review, see Chatfield and Collins, "Introduction to Multivariate Analysis,"
       published 1980 by Chapman and Hall, New York; and Höskuldsson Agnar, "Predictions
       Methods in Science and Technology," published 1996 by John Wiley and Sons, New
       York). Multivariate data analyses are used for a variety of applications involving these
       multiple factors, including quality control, process optimization, and formulation
25   determinations. The analyses can be used to determine whether there are any trends in
       the data collected, whether the properties or responses measured are related to one
       another, and which properties are most relevant in a given context (for example, a disease
       state). Software for statistical analysis is commonly available, e.g., from Partek Inc. (St.
       Peters, MO; see www.partek.com).

30   Multivariate statistics is particularly useful for determination and analysis
       of polygenic effects within a cell line. One common method of multivariate analysis is
       principal component analysis (PCA, also known as a Karhunen-Loève expansion or
       Eigen-XY analysis). PCA can be used to transform a large number of (possibly)
       correlated variables into a smaller number of uncorrelated variables, termed "principal
35   components." Multivariate analyses such as PCA are known to one of skill in the art, and
       can be found, for example, in Roweis and Saul (2000) Science 290:2323-2326 and
       Tenenbaum et al. (2000) Science 290:2319-2322.

5   The responses generated by a given plurality of cell lines can be grouped, or clustered, using multivariate statistics. Clusters for each different stimulation (treating) and observation (detecting) experiment are compared and a secondary set of correlations/noncorrelations are made. Based on these different sets of correlations, a network map can be created wherein the relative relationships of the different genetic

10   elements can be established as well as how they may act in concert. In addition, the data can be visualized using graphical representations. Thus, the temporal changes exhibited by the different biochemical and genetic elements within a genetically-related group of cells lines can be transformed into information reflecting the functioning of the cells within a given environment.

15   Compounds that evoke a similar genetic response are likely to share one or more mechanisms of action. Through analysis of a set of compounds and/or chemical analogues, pathway specific inhibitors and comparable pharmacophores, the mechanistic differences and commonalities can be elucidated. A difference analysis provides the means to identify one or more elements responsible for the desired activity or phenotypic

20   response. In addition, the dose response data coupled with the difference analysis enables the creation of a mechanism of action (MOA) model. Libraries of compositions can be screened for their ability to evoke a genetic response profile similar to that targeted for the desired activity. Furthermore, compositions can be tested against the MOA model to assess if they stimulate similar mechanisms of response.

25   As a final step in the methods of identifying a new composition with a desired activity, a second set of compositions, or library of compositions, is screened by determining the genetic response profiles for member components. Optionally, the genetic profile is determined in a manner similar to that used for the first set of compositions. However, the number of genetic responses determined need not be the

30   same as those determined for the first set of composition; a selected subset of responses, for example, responses related or correlating to the desired activity being identified, can be monitored.

        Additional experimentation can be performed that would aid in the identification of specific genes that, for example, confer sensitivity or resistance to drug

35   treatment. Knowledge of these genes and/or mechanisms can assist in the search for patient segregation markers and surrogate clinical endpoints. As one example,

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5   toxicological studies can be performed concomitant with or in addition to screening of
       compositions for the desired activity.

                    The following examples are offered for illustration. One of skill in the art
       will recognize that alternative desired activities can be selected, and a variety of
       noncritical parameters can be changed.

10   EXAMPLE 1: DEVELOPMENT OF CHEMOTHERAPEUTICS FOR CANCER
       TREATMENT

                    The methods of the present invention can be used in the development of
       novel chemotherapeutics for cancer treatment. The methods employ one or more
       modified cancer cell lines prepared as follows. One or more cancer cell lines are selected

15   and challenged with a chemotherapeutic agent (e.g. methotrexate or cisplatin), and
       allowing the cells to grow. Different dosing techniques may be used, for example,
       increasing the dosage of the agent over multiple cell cycles, using multiple doses of the
       same concentration over multiple cycles, or just using a single dose of the agent.
       Modified cells that are capable of growth in the dosed environment are selected. These

20   modified cells have developed a resistance to the particular compound, i.e. they have a
       different response to the primary activity of the compound versus the parent cell line.
       Cells that survive the challenge with the chemotherapeutic agent can be individually
       selected and grown clonally for inclusion in the plurality of cell lines. Optionally, the
       new cell line is treated with the chemotherapeutic agent to confirm its resistance.

25   EXAMPLE 2: GENERATION OF APOPTOSIS-MODIFIED CELL LINES

                    The methods of the present invention can also be used to identify novel apoptosis
       inducers and/or apoptosis inhibitors. For these methods, the plurality of cell lines
       includes cells that are capable of surviving a pro-apoptosis event. The cells are generated,
       for example, by treating a cell line with a protein that strongly induces apoptosis, and

30   selecting the cells that survive the treatment. For example, the Fas ligand (which binds
       to Fas receptor) induces apoptosis in Jurkat cells, a process which can be monitored by
       flow cytometry. A common apoptosis assay is the Annexin V assay that measures
       disturbance and inversion of the outer cellular membrane. The vast majority of cells
       treated with Fas ligand will transition into apoptosis; however, within the cell culture, a

35   small population of cells will resist going into apoptosis. These modified cells can be
       selectively sorted from the general population using flow cytometry, based on being

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5   negative for the Annexin V marker. Alternatively, the modified cells can be selected by
     subjecting the population to a survival selection screen, such as known to one of skill in
     the art.

             The modified cells have undergone some alteration that prevents the induction of
     apoptosis. Examples of the types of alterations that may result in survival include
10   mutation of the Fas receptor, strong down regulation of Fas receptor, mutation or down
     regulation of one of the proteins in the pathway downstream from the receptor, including
     one of the caspase proteins, or induction of a pathway that is anti-apoptotic with respect
     to cell regulation. The modified cells are then included in the plurality of cell lines of the
     methods of the present invention.

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EXAMPLE 3: IDENTIFICATION OF NOVEL ANTI-CANCER COMPOUNDS BASED UPON NA+K+-ATPase INHIBITORS

             Na^+K^+-ATPase (sodium pump) is an ion transporter present in the
     membrane of most eukaryotic cells and either directly or indirectly controls many
20   essential cellular functions (Blanco and Mercer (1998) "Isozymes of the Na-K-ATPase:
     heterogeneity in structure, diversity in function" Am J Physiol 275:F633-50). For
     example, Na^+K^+-ATPase activity affects intracellular Ca^{2+} levels and modulates gene
     expression (e.g., androgen receptor) and apoptosis (Bortner et al. (1997) "A primary role
     for K+ and Na+ efflux in the activation of apoptosis" J Biol Chem 272(51):32436-42;
25   Furuya et al. (1994) "The role of calcium, pH, and cell proliferation in the programmed
     (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin"
     Cancer Res 54(23):6167-75), and is modulated by insulin, protein kinases (A, C), cAMP
     and other second messengers (Haas et al. (2000) "Involvement of Src and epidermal
     growth factor receptor in the signal-transducing function of Na+/K+-ATPase" J Biol
30   Chem 275(36):27832-7; Huang et al. (1997) "Differential regulation of Na/K-ATPase
     alpha-subunit isoform gene expressions in cardiac myocytes by ouabain and other
     hypertrophic stimuli" J Mol Cell Cardiol 29(11):3157-67; Manna et al. (2000) "Oleandrin
     suppresses activation of nuclear transcription factor-kappaB, activator protein-1, and c-
     Jun NH2-terminal kinase" Cancer Res 60(14):3838-47; Kometiani et al. (1998) "Multiple
35   signal transduction pathways link Na+/K+-ATPase to growth-related genes in cardiac
     myocytes: The roles of Ras and mitogen-activated protein kinases" J Biol Chem
     273(24):15249-56; Sweeney and Klip (1998) "Regulation of the Na+/K+-ATPase by

                                          26

5   insulin" Mol Cell Biochem 182:121-33, Xie et al. (1999) "Intracellular reactive oxygen
     species mediate the linkage of Na+/K+-ATPase to hypertrophy and its marker genes in
     cardiac myocytes" J Biol Chem 274(27):19323-8). Regulation of this enzyme and its
     individual isoforms may play a key role in the etiology of some pathological processes
     including, but not limited to, cardiovascular, neurological, renal, and metabolic diseases

10   purported to involve dysfunction of Na$^+$K$^+$-ATPase activity (see, for example, Akopyanz
     et al. (1991) "Tissue-specific expression of Na,K-ATPase beta-subunit" FEBS Lett 289(1):
     8-10; Blok et al. (1999) "Regulation of expression of Na+,K+-ATPase in androgen-
     dependent and androgen-independent prostate cancer" Br J Cancer 81(1):28-36;
     McDonough and Farley (1993) "Regulation of Na,K-ATPase activity" Curr Opin Nephrol

15   Hypertens 2(5):725-34; and Rose and Valdes (1994) "Understanding the sodium pump
     and its relevance to disease" Clin Chem 40(9):1674-85). Furthermore, changes in Na$^+$K$^+$-
     ATPase activity may play a role in certain cancers.

          The sodium pump is made up of two predominant subunits, a catalytic α
     subunit and a β subunit that is required for activity. In addition, a third γ subunit has been

20   found in renal cells. The β subunit also functions in cell-cell interactions and in the
     intracellular transport of the α subunit to the membrane. Each major subunit has several
     isoforms (e.g., α1, α2, α3, α4 and β1, β2, β3) that show a tissue-specific pattern of
     expression, which is regulated by the mineralcorticoid and glucocorticoid receptors. For
     example, the β1-subunit is down-regulated by androgen and increased in androgen

25   insensitive prostate cancer cells.

          Inhibition of the Na$^+$K$^+$-ATPase has an anti-cancer effect in breast cancer
     clinical studies and various cancer cell lines (Haux (1999) "Digitoxin is a potential
     anticancer agent for several types of cancer" Med Hypotheses 53(6):543-8). Furthermore,
     the chromosomal location of the gene encoding the β1 subunit is located in the same

30   region as the prostate cancer sensitivity locus, HPC1. In light of the anticancer activity
     of Na$^+$K$^+$-ATPase inhibitors (e.g. a desired effect secondary to the cardiac), Na$^+$K$^+$-
     ATPase is a potential cancer drug target. Novel compositions having an increased anti-
     cancer activity but with the same or, preferably, a decreased ATPase inhibitory activity,
     can be identified using the methods of the present invention.

Selection of Initial Set of Compositions
                    The sodium pump is the only known receptor for the cardiac glycosides,
potent inotropic drugs used in the treatment of congestive heart failure (Hauptman and
Kelly (1999) "Digitalis" Circulation 99:1265-70). Endogenous ligands structurally similar
to digitoxin or ouabain may control the activity of this important molecular complex *in*
10   *vivo*. Digitoxin and ouabain have also been implicated as potential anti-cancer drugs
based on clinical studies and selective effects on normal versus tumor cells (10, 30, 31,
33). These and related compounds are specific inhibitors of the membrane-bound Na^+K^+-
ATPase responsible for regulating $Na^{+/}K^+$ exchange (and, as a consequence, intracellular
Ca^{2+} levels).

15   Analysis of clinical trial data indicates that five years after mastectomy,
women on digitalis had a 9.6-fold reduction in recurrence of breast cancer (Haux, *ibid.*). It
has also been shown that digitalis (30-60 nM) affects cell adherence and induces
apoptosis in several Glioblastoma cell lines. The drug tamoxifen also appears to inhibit
the Na^+K^+-ATPase (in addition to the estrogen receptor, ER) as part of its anti-cancer
20   action (see Repke and Matthes (1994) "Tamoxifen is a Na(+)-antagonistic inhibitor of
Na+/K(+)-transporting ATPase from tumour and normal cells" J Enzyme Inhib 8(3):207-
12) and is known to have an anti-cancer effect in ER- cancers (e.g., melanoma,
glioblastoma).

                    Androgens are required for prostate development, growth and
25   differentiation, and maintenance of function in the adult. Androgen action is mediated by
the androgen receptor (AR), an androgen-dependent transcription factor and member of
the nuclear receptor family (which includes receptors to steroids, retinoids, thyroid
hormone, and Vitamin D). The AR pathway up-regulates as well as down-regulates
numerous factors that affect the growth, differentiation, and survival of prostate epithelial
30   and cancer cells. Androgen insensitivity is one of the major clinical problems in treating
prostate cancer (12).

                    There are several possible functional connections between the Androgen
Receptor and the Na^+K^+-ATPase.   The gene encoding the β-1 subunit of Na^+K^+-ATPase
is down-regulated in the presence of androgens. Expression is high in androgen-
35   independent cells and low in androgen-dependent cells (grown in the presence of
androgens). Down-regulation induced by androgen reduces Na^+K^+-ATPase in the
membrane. In androgen-dependent cells, a ouabain-induced decrease in Na^+K^+-ATPase

5   activity reduces sensitivity of these cells to cisplatin. However, an androgen-induced decrease in Na^+K^+-ATPase activity does not protect cells against cisplatin.

        Partial inhibition of Na^+K^+-ATPase by ouabain increases intracellular Ca^{2+} levels and the expression of c-fos, c-jun, and the transcription factor AP-1. Ca^{2+} mobilizers repress AR-mediated induction of PSA and hKLK2 by inhibiting AR trans-

10   activation activity by AP-1 proteins. Androgen deprivation can induce the elevation of intracellular Ca^{2+}, the expression of AP-1 genes (c-fos, c-jun), and apoptotic cell death.

<u>Selection of Cell Lines</u>

        A number of different cell lines have demonstrated differences in their responsiveness to the describes compositions, their primary activities and apoptosis. For

15   example, digitalis (at non-toxic doses) induces apoptosis in Jurkat (T-cell) and Daudi (B-cell) cell lines, but not in K562 (erthroleukemia cell) lines. Other studies have shown that ouabain sensitizes malignant (but not normal) cells to irradiation (Verheye-Dua and Bohm 1998 "Na+, K+- ATPase Inhibitor, Ouabain Accentuates Irradiation Damage in Human Tumour Cell Lines" Radiation Oncology Investigations 6:109-119).

20   A number of cell matrices can be selected for their differential response and modeling of prostate cancer. For example, BPH (benign prostatic hyperplasia) cells are commonly used as the "normal" control cell line. PC3 and DU145 cells (parent lines) have lost AR expression and are unresponsive to androgen treatment. In addition they have high doubling times and represent aggressive cancer growth. These same cell lines,

25   if transfected with a vector expressing androgen receptor protein (modified lines), become responsive to androgen treatment.

        Complementing the androgen insensitive lines are LNCap, MDA-PCA 2a, 2b, and ARCaP. LNCap expresses AR and is androgen responsive. The MDA-PCa lines overexpress a mutated AR. They have adapted the AR pathway to be able to grow, but

30   with a lower doubling time and are less aggressive than PC3 and DU145. These mutant lines represent loss of activity because of one or more of the following types of adaptations, change in ligand specificity, AR amplification, AR ligand-independent activation, and/or coactivator amplification and co-repressor downregulation. The ARCaP line expresses AR and is growth inhibited upon androgen treatment. This cell

35   line is capable of bypassing the AR pathway for its growth, using one or more of the following mechanisms, activation of other oncogenes or inactivation of tumor suppressor

5   genes (e.g., LNCaP transfected with Ras or Bcl-2), AR mutations and deletions, and/or
AR gene inactivation by DNA methylation.

Treatment of these and other like cell lines with the described
compositions and possibly others, can be used to generate multiple response profiles and
enable the differentiation of activities associated with Na^+K^+-ATPase interaction, AR
10   interaction and proapoptotic events. The identified profiles and/or patterns within the
response profiles can then be used as target profiles in the screen of compound libraries to
identify those compounds with preferred profiles correlating to related proapoptotic
activity while minimizing interacting with Na^+K^+-ATPase and AR.

EXAMPLE 4: IDENTIFICATION OF NOVEL APOPTOSIS INDUCERS AND
15   ## SELECTION OF TREATMENT-SENSITIVE POPULATIONS

In addition to identifying novel compositions for treatment of disease
states, the genetic response profiles of the present invention can be used to select patients
within a population who have a significantly higher probability of responding to treatment
with a therapeutic composition. For example, application of cell culture techniques,
20   bioinformatics, and high throughput screening can be used to generate response profiles
that predict a probability of clinical efficacy of a drug composition or library of
compositions.

The present invention provides methods of identifying organisms that are
sensitive to treatment with a drug composition. The methods include the steps of
25   identifying a set of genetic response markers, e.g. one or more genes, RNA sequences,
proteins, metabolites, phenotypes and the like, and a correlating genetic response profile
for a biochemical process or disease state for which the drug composition is used as
treatment; providing a plurality of cell lines, wherein the plurality of cell lines comprises
at least one modified cell line which differs from a corresponding parent cell line in its
30   sensitivity to the drug composition; determining a first set of genetic response profiles
that potentially indicate drug resistance by a) treating each member of the plurality of cell
lines with the drug composition; and b) monitoring the set of genetic response markers;
comparing the first set of genetic response profiles to clinical data for a first population of
organisms, thereby identifying a pattern of responses correlating to sensitivity to
35   treatment with the drug composition; and generating a second set of genetic response
profiles for members of a second population of organisms and screening the second set of

5   genetic response profiles for the pattern of responses correlating to sensitivity, thereby
      identifying organisms that are sensitive to treatment with the drug composition.

                    The present example describes the use of genetic response profiles to
      identify organisms which will respond better to treatment with an apoptosis inducer (AI)
      (for example, a bisphosphonate class therapeutic composition), using gene expression for
10   multiple genes as the genetic response markers. In brief, a number of genes which
      correlate to key expression response markers of apoptosis are identified. AI-based
      genetic response profiles are then determined using an *in vitro* model of differential
      response to AI for a plurality of drug-susceptible and drug-resistant cancer cell lines. The
      genetic response profiles are compared to profiles from clinical samples, to correlate
15   response pattern with clinical outcome. Ultimately, the genetic response patterns are used
      to analyze patient-derived cells, thereby predicting the likelihood that the patient will
      respond to treatment with the apoptosis inducer.

                    Apoptosis and Cancer
                    Cancer develops through a variety of mechanisms including, but not
20   limited to, the functional failure of multiple gene combinations. Because of the range of
      genes potentially affected in a given cancer, it is unlikely that any single therapeutic will
      impact every cancer types. As a consequence, only a portion of a given patient
      population will preferentially respond to each treatment. It is desirable to model cancer
      heterogeneity and to visualize how a particular therapeutic affects these cells, linking
25   expression response to phenotypic outcome, and ultimately, clinical outcome. Use of
      these expression response patterns enables the identification and/or selection of a subset
      of the patient population with an increased likelihood of response to a particular
      therapeutic.

                    One approach to generation of the genetic response profiles is to sample
30   blood and tumor tissue from a large population of cancer patients (>1000) who have been
      treated with an apoptosis-inducing composition. Generally, it is very difficult and costly
      to obtain access to the large sample population necessary to capture statistically
      significant differences attributable to inducer activity, independent of the genetic
      heterogeneity that naturally occurs among individuals but is unrelated to the disease and
35   treatment. Therefore, an *in vitro* cell-culture model is employed to generate genetic
      response profiles and capture many of the statistically significant differences among
      cancer types. While the cell culture model might not identify all of the possible

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5　　mechanisms of clinical response, it is likely to be predictive for a large percentage of the
population. Likewise, the model can be used to identify those individuals who are
unlikely to respond to treatment. Additionally, an *in vitro* process is far more cost
efficient and can be performed quickly while delivering the high level of accuracy in the
data necessary for modeling.

10　　　　　　　　　Selection of Marker Genes

　　　　　　　The first step in the methods of the present invention involves performing
experiments to screen for genes that are responsive to AI treatment. These genes include
a broad spectrum of gene types, including those that are directly influenced by AI, genes
associated with AI response (e.g. apoptosis genes), as well as a number of genes known to
15　　play a role in cancer. Optionally, about 1000 genes are screened, to identify the key
responders to AI over a variety of cell types. This data will be used to identify the set of
genes that correlate to expression response markers of apoptosis.

　　　　　　　In one embodiment, the samples are monitored at the RNA level using
microarrays. In another embodiment, the samples are analyzed at the protein level using
20　　2-dimensional gel electrophoresis and mass spectrometry.

　　　　　　　Approximately 10 different cancer-related cell lines are provided for the
study. These lines include cells types that are known *in vivo* targets for and other AI
agents as well as a diversity of potential target tissue types for these therapeutics.
Exemplary cancer-related cell lines include: PC-3 (prostate cancer), HepG2 (liver cancer),
25　　HL-60 (leukemia), A-549 (lung cancer), MCF-7 (breast cancer), SW620 (colon cancer),
Saos-2 (osteosarcoma), MG-63 (osteoblasts), caco-2 (colon cancer), and PA-1 (ovarian
cancer).

　　　　　　　The cell lines are exposed to the AI, and genes involved in AI response are
identified. Cellular and genetic responses are monitored in response to AI treatment for
30　　the broad spectrum of cell lines included in the plurality of cell lines. The data
(optionally along with other data generated using different chemical compositions for
same cell lines) can be used to cluster gene responses and map the genes into a number of
categories, including, but not limited to, general expression responders, AI specific
responders, disease/cell specific responders, and nonresponders. The identified genes
35　　capture the cell response mechanisms for AI treatment. The genes will be used to create
an optimal gene set for use in generation of genetic response profiles.

Generation of Genetic Response Profiles and Identification of AI Sensitivity Patterns

The genetic response profiles generated for the cancer lines are used to design the desired expression response pattern that can be used to monitor additional organisms (i.e., patients) and determine a probability of response to AI. Optionally, an *in vitro* model for mechanisms of AI sensitivity and resistance is prepared using both AI-resistant and AI-sensitive cell lines. The cell lines are generated, for example, from the cell lines analyzed during identification of the genetic response markers. One or more of these cell lines can be used as parent cell lines for the development of multiple resistant daughter lines.

The development of daughter resistant cell lines (modified cell lines) for each parent line involves treatment of parent lines with AI and taking the cells through a selection process. Because the targeted endpoint for susceptibility is cell death, cell survival can be used as a selection tool. Cell lines are treated with AI and surviving cells are cultured. These surviving cells are optionally subjected to 1-2 additional rounds of selection in order to reduce leakage of susceptible cells. From these surviving cells a number of single clones are selected and grown in individual culture. Isolation of single cells and confirmation of their drug resistance is optionally performed by cell sorting flow cytometry. Anywhere from about 10 to about 50 clones are developed and maintained as separate cell lines. One advantage to selecting and using multiple clones is the generation of various modifications leading to resistance (because it is likely that cell survival during treatment will occur through a number of mechanisms). Therefore it is possible to create multiple resistant cell lines that represent several potential resistance mechanisms.

By using genetically-related, parent (sensitive) and daughter (resistant) cell lines representing a number of cancer types, the genes that are specifically responsible for affecting the potency and efficacy of AI can rapidly be determined. Furthermore, the genetic relationship of parent and daughter (modified) cell lines eliminates much of the gene expression variability that is found in unrelated samples, simplifying gene identification, and greatly increasing the correlation between AI and genetic mechanisms that impact its efficacy.

For example, about 2-4 cancer cell lines representative of the cancer types targeted for treatment are selected from the previously tested group, and treated with AI to develop multiple AI resistant lines for each parent cell line. Optionally, a total of about

5   96 cell lines are generated in this manner. This plurality of cell lines is used to characterize the differential gene expression response in sensitive parent and resistant daughter lines plus and minus exposure to AI. Additionally, a statistical analysis of the expression patterns is performed to identify genes and gene response patterns that indicate the level of AI sensitivity. These experiments provide both a database of expression

10   response patterns for comparative analysis (the first set of genetic response profiles) and the optimal gene set for use in screening patient samples, and for screening and identifying new AI compounds.

For each of the parent and resistant daughter cell lines a gene expression pattern, e.g., a genetic response profile, is determined. The profiles are generated for both

15   AI treated and untreated cultures. Differential parent/daughters expression patterns within each cell line can be determined. A comparison or clustering of different parent/daughter patterns enables a detailed mapping of patterns representative of different mechanisms of resistance. The more parent/daughter patterns generated, analyzed and compared, the higher the level of statistical confidence.

20   An additional analysis among cell lines can also be performed. These comparisons enable one to visualize consensus patterns that represent resistance mechanisms to AI and to identify resistance mechanisms that may be tissue or cancer-type specific. Conversely, patterns exclusive and universal to parent lines will provide a diagnostic for AI susceptibility. Following this analysis, all of these patterns as

25   represented in a database can be used to evaluate clinical samples in the next step of the methods of the present invention, optionally using the same or similar gene expression tools. In addition, this database may be used to identify new compounds that are AIs but are not susceptible to the same mechanisms of drug resistance.

Clinical Correlation Studies

30   The methods of the present invention include the step of generating a second set of genetic response profiles for members of a second population of organisms and screening the second set of genetic response profiles for the pattern of responses correlating to sensitivity, thereby identifying organisms that are sensitive to treatment with the drug composition. In one embodiment, the second population of organisms

35   includes clinical samples. A retrospective study to correlate response patterns with clinical outcome assists in the identification of desired patterns of response and in the

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5   screening of the second population. The results from screening the second population can also be used to further refine the predictive potential of the pattern analysis.

The methods of the present invention provide a wealth of data, response patterns, methods for obtaining and analyzing samples, and bioinformatic techniques for the analysis of data and determination of therapeutic candidates with improved activity

10   profiles and efficacy probabilities once they are in the preclinical or clinical setting.   All of which can be used in an ongoing basis to determine a probability that a drug composition will be effective in treating a disease and each individual patient who has the disease. As a consequence it is fully expected that the genetic response profiles and patterns generated via the methods of the present invention can be used to identify

15   compositions with improved therapeutic characteristics and those individuals with the highest probability of responding to a given drug composition.

USES OF THE METHODS, DEVICES AND COMPOSITIONS OF THE PRESENT INVENTION

Modifications can be made to the methods and materials as described

20   above without departing from the spirit or scope of the invention as claimed, and the invention can be put to a number of different uses, including:

The use of any method herein, to identify novel compositions.

The use of any method herein, to identify populations which will preferably respond to a composition having a desired activity.

25   An assay, kit or system utilizing a use of any one of the selection strategies, materials, components, cell matrices, methods or substrates hereinbefore described. Kits will optionally additionally include instructions for performing the methods or assays, packaging materials, one or more containers which contain assay, device or system components, or the like.

30   In a further aspect, the present invention provides for the use of any component or kit herein, for the practice of any method or assay herein, and/or for the use of any apparatus or kit to practice any assay or method herein.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a

35   reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the present invention. For example, all the methods and compositions described above may be used in various combinations. All of the

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5   compositions and/or methods disclosed and claimed herein can be made and executed
    without undue experimentation in light of the present disclosure. While the compositions
    and methods of this invention have been described in terms of preferred embodiments, it
    will be apparent to those of skill in the art that variations may be applied to the
    compositions and/or methods, and in the steps or in the sequence of steps of the method
10 described herein without departing from the concept, spirit and scope of the invention.
    More specifically, it will be apparent that certain agents which are both chemically and
    physiologically related may be substituted for the agents described herein while the same
    or similar results would be achieved. All such similar substitutes and modifications
    apparent to those skilled in the art are deemed to be within the spirit, scope and concept of
15 the invention as defined by the appended claims. All publications, patents, patent
    applications, Internet citations, and/or other documents cited in this application are
    incorporated by reference in their entirety for all purposes to the same extent as if each
    individual publication, patent, patent application, Internet citation and/or other document
    were individually indicated to be incorporated by reference for all purposes.